Biochimica et Biophysica Acta 1853 (2015) 1073–1086

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

NLRX1 acts as tumor suppressor by regulating TNF-α induced apoptosis and metabolism in cancer cells

Kritarth Singh [a,b], Anastasia Poteryakhina [c], Andrei Zheltukhin [c], Khyati Bhatelia [a,b], Paresh Prajapati [a,b], Lakshmi Sripada [a,b], Dhanendra Tomar [a], Rochika Singh [a], Arun K. Singh [a], Peter M. Chumakov [c,*], Rajesh Singh [a,**]

[a] Department of Biochemistry, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India
[b] Department of Cell Biology, School of Biological Sciences and Biotechnology, Indian Institute of Advanced Research, Gandhinagar, India
[c] Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow, Russia

ARTICLE INFO

Article history:
Received 24 September 2014
Received in revised form 23 December 2014
Accepted 21 January 2015
Available online 30 January 2015

Keywords:
NLRX1
Caspase-8
Mitochondria
Metabolic reprogramming
Inflammation
Cancer

ABSTRACT

Chronic inflammation in tumor microenvironment plays an important role at different stages of tumor development. The specific mechanisms of the association and its role in providing a survival advantage to the tumor cells are not well understood. Mitochondria are emerging as a central platform for the assembly of signaling complexes regulating inflammatory pathways, including the activation of type-I IFN and NF-κB. These complexes in turn may affect metabolic functions of mitochondria and promote tumorigenesis. NLRX1, a mitochondrial NOD-like receptor protein, regulate inflammatory pathways, however its role in regulation of cross talk of cell death and metabolism and its implication in tumorigenesis is not well understood. Here we demonstrate that NLRX1 sensitizes cells to TNF-α induced cell death by activating Caspase-8. In the presence of TNF-α, NLRX1 and active subunits of Caspase-8 are preferentially localized to mitochondria and regulate the mitochondrial ROS generation. NLRX1 regulates mitochondrial Complex I and Complex III activities to maintain ATP levels in the presence of TNF-α. The expression of NLRX1 compromises clonogenicity, anchorage-independent growth, migration of cancer cells in vitro and suppresses tumorigenicity in vivo in nude mice. We conclude that NLRX1 acts as a potential tumor suppressor by regulating the TNF-α induced cell death and metabolism.

1. Introduction

Clinical and experimental studies suggest that inflammation is intricately linked with tumorigenesis. In colorectal, hepatic, breast and several other cancer types, an inflammatory condition may precede the development of malignancy [1–3]. For example, inflammatory bowel disease (IBD) is associated with colon cancer and an infection by *Helicobacter pylori* progressively leads to gastric carcinoma [3,4]. However, despite the numerous examples of the apparent association of chronic inflammatory conditions with higher incidences of cancer, the molecular mechanisms linking these pathologies are still not well understood.

Inflammation, irrespective of its origin, promotes cell survival, proliferation of malignant cells and conditions the tumor microenvironment for further metastasis. Emerging clinical reports suggest that the levels of specific cytokines are altered in patients with different cancer types including breast, gastric, colorectal and hepatocellular carcinomas [5]. Increased levels of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), macrophage migration inhibitory factor (MIF), transforming growth factor beta (TGF-β), interleukins-6, -8, -10 and -18 (IL-6, IL-8, IL-10 and IL-18) were reported in patients with advanced-stage pancreatic, colorectal and breast cancers [6–15]. Serum levels of TNF-α were elevated in eight independent types of cancer including breast, colorectal and gastric carcinomas [5,9,13]. In tumor microenvironment, TNF-α secreted by tumor cells or by inflammatory cells, promotes tumor cell survival through the stimulation of NF-κB pathway [16]. The activation of NF-κB up-regulates the expression of genes stimulating cell cycle progression and promotes epithelial–mesenchymal transition [17]. The binding of TNF-α to Type I TNF receptor (TNFR1) results in a pro-survival stimulation of NF-κB, through the formation of proximal plasma membrane bound complex I consisting of TNF receptor-associated protein with death domain (TRADD), receptor-interacting protein 1 (RIP1) and TNF receptor-associated factor 2 (TRAF2). During the TNF-α induced apoptosis, the complex-I dissociates from TNFR1 and recruits the Fas-associated death domain (FADD) and Caspase-8, forming cytosolic complex-II, where Caspase-8 is activated, which further initiates the downstream proteolytic cascade

* Correspondence to: P.M. Chumakov, Cell Proliferation Laboratory, Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Vavilov Street 32, Moscow, Russia. Tel.: +7 499 135 2331; fax: +7 499 135 1405.
** Correspondence to: R. Singh, Department of Biochemistry, Faculty of Science, The M.S. University of Baroda, Vadodara, Gujarat, India. Tel.: +91 265 2795594.
E-mail addresses: peter@chumakov.com (P.M. Chumakov), singhraj1975@gmail.com (R. Singh).

http://dx.doi.org/10.1016/j.bbamcr.2015.01.016
0167-4889/

[18,19]. The emerging evidences suggest that Caspase-8 may translocate to different subcellular sites including mitochondria in response to different stimuli [20–22]. TRADD, TRAF2 and RIP1 subunits of complex-II together with Pro-caspase-8 have been reported to translocate to mitochondria during the TNF-α induced apoptosis [23]. The functional relevance of Caspase-8 and other subunits of complex-II translocation to mitochondria is not clear.

Stimulation of the cells with TNF-α induces reactive oxygen species (ROS) generation however the regulatory mechanisms in different pathological conditions are still not clear. The mitochondrial Complex I and Complex III are the major sites of ROS generation [24]. Impaired Complex I and Complex III activities alter mitochondrial integrity by decreasing membrane potential, ATP synthesis and oxygen consumption [25]. The alteration of mitochondrial functions in response to TNF-α shifts the cancer cell metabolism toward glycolysis [26] however the mechanisms are not well understood. Upregulated glycolysis is a characteristic metabolic hallmark of cancer cells that is required to meet the bioenergetic demands. Thus, TNF-α signaling may confer a selective advantage to cancer cells for survival and proliferation through constitutive activation of NF-κB and reprogramming of metabolism. However, molecular mechanisms for the TNF-α-mediated regulation of mitochondrial bioenergetic function remains elusive.

Mitochondria are emerging as a critical signaling platform for the assembly of signallosomes regulating the inflammatory pathways [27]. Mitochondrial antiviral signaling protein (MAVS) localizes on the outer membrane of mitochondria and serves as an adaptor protein for RIG1, recognizing dsRNA during viral infections. MAVS is essential for the type-I IFN and NF-κB activation during viral infections [28]. Recent studies have suggested that COX5B, a subunit of Cytochrome c Oxidase (CcO) complex negatively regulates antiviral innate immunity by inhibiting MAVS signallosomes at the mitochondria [29]. Similarly, mediator of IRF3 activation (MITA) is a mitochondria-associated ER membrane (MAM) protein located in the area of close juxtaposition between mitochondria and ER. MITA is a crucial regulator of IFN and NF-κB signaling during infection by DNA viruses [30]. The study from our lab suggests that MITA is also an important regulator of inflammation acting as a potential tumor suppressor in breast cancer [31]. Similarly, a highly conserved NOD-like receptor with a mitochondria localization signal, the nucleotide-binding domain (NBD) and leucine-rich repeat (LRR)-containing family member X1 (NLRX1) [32] is an important component of TLR mediated inflammatory pathways [33]. The role of the NLRX1 in regulating the cross talk of inflammation and mitochondrial function and its involvement in carcinogenesis is not well understood.

In the present work, we identify NLRX1 as a crucial regulator of TNF-α induced cell death and reveal its potential role as a tumor suppressor. We report here that NLRX1 sensitizes cells to the TNF-α induced apoptosis by potentiating Caspase-8 at the level of complex-II. NLRX1 contributes to the TNF-α induced generation of ROS by regulating the activities of mitochondrial respiratory Complex I and Complex III. NLRX1 negatively regulates clonogenic survival of tumor cells in vitro and tumor growth in vivo by modulating oxidative phosphorylation and metabolism.

2. Materials and methods

2.1. Cell culture and reagents

HEK293, HeLa, and MCF-7 cells were cultured in Dulbecco's modified Eagle's media (DMEM, Life Technologies, Carlsbad, CA, USA). T47D and MDA-MB-231 cells were cultured in RPMI 1640 (Life Technologies, USA) and Leibovitz's L-15 media (HI-MEDIA, India) respectively. Media were supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Life Technologies), 1% penicillin, streptomycin and neomycin (PSN) antibiotic mixture (Life Technologies). Full length NLRX1 (isoform 1) cloned into pcDNA3.1 vector was provided by Dr. S E Girardin (University of Toronto, Ontario, Canada). Full length TRIM13 and HA-TRAF2 were kind gifts from Dr. Olle Sangfelt (Department of Oncology/Pathology, Cancer Centrum Karolinska, Stockholm, Sweden) and Dr. S M Srinivasula (IISER, Thiruvananthapuram, Kerala, India), respectively. The primary antibodies used were NLRX1, β-actin and c-MYC (Abcam, Cambridge, UK), PARP, Caspase-8 (Cell Signaling Technology, Inc., USA) and anti-HA peroxidase (Roche Applied Science, IN, USA). HRP-conjugated anti-mouse and anti-rabbit antibodies (Thermo Scientific, Inc., IL, USA) were used. TNF-α (Tumor Necrosis Factor-alpha) and Mito-TEMPO were procured from Enzo Life Sciences, Inc. USA. Cyclohexamide, antimycin, rotenone, H_2O_2, oligomycin and N-Acetyl Cysteine (NAC) were purchased from Sigma-Aldrich, USA. z-VAD-fmk (N-Benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone) (BioVision, Inc., CA, USA), IETD-fmk (Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethyl ketone) (Clontech Laboratories, Inc., USA) were used. CM-H_2DCFDA and MitoSOX™ Red were purchased from Molecular Probes Inc., USA.

2.2. Cell death and proliferation assay

Cell death and proliferation was quantified by Trypan blue exclusion assay and MTT assay respectively as previously described [31].

2.3. Generation of stable cell lines

MT-RFP stable cell line in MCF-7 was generated as previously described to study mitochondrial localization of NLRX1 [34]. Similarly, stable knockdown of NLRX1 were generated in HEK293 and HeLa cells. Cells were transfected with NLRX1 shRNA and control shRNA and stable clones were selected and maintained in puromycin (3 μg/ml) containing DMEM.

2.4. Immunoprecipitation and Western blotting

For immunoprecipitation, HEK293 cells were plated at a density of 2×10^6 cells/90 mm^2 dish and transfected with indicated constructs using a calcium phosphate transfection method. After 24 h of transfection, the cells were treated with TNF-α/CHX for an indicated time interval, thereafter the cells were washed with cold DPBS (Life Technologies) and harvested. Cells were lysed in NP40 IP lysis buffer (100 mM NaCl, 50 mM Tris–HCl, 10% Glycerol, 0.1% Nonidet P-40) containing complete protease inhibitor cocktail (Roche Applied Science, IN, USA) and incubated on ice for 1 h and centrifuged at 13,000 rpm for 15 min at 4 °C. Supernatant was collected and incubated overnight with Anti-HA Affinity Matrix on a roller shaker at 4 °C. The beads were washed three times with NP40 IP lysis buffer, resuspended in 5 × SDS-PAGE sample buffer, resolved on 12% SDS-PAGE and analyzed by Western blotting using specific antibodies.

For Caspase-8 and Caspase-3 immunoblotting, HEK293 cells were plated at a density of 5×10^5 cells/well in a 6-well plate and transfected with indicated constructs using calcium phosphate transfection method. After 24 h of transfection, the cells were treated with indicated reagents for specific time. The cells were harvested in cold PBS and lysed in RIPA lysis buffer (150 mM NaCl, 50 mM Tris–HCl, 1% Triton X-100, and complete protease inhibitor cocktail (Roche, Germany), and analyzed by Western blotting as indicated above. Western blot quantification was performed by densitometry using Image J 1.45 software (NIH, MD, USA).

2.5. Caspase-8 luciferase assay

Caspase-8 activation assay was performed using Caspase-Glo® 8 Assay Systems (Promega, USA) according to manufacturer instructions as described previously [35].

2.6. Confocal microscopy

Mitochondrial localization of NLRX1 was monitored by confocal microscopy. Briefly, MT-RFP-MCF-7 stable cell line were plated on cover slip at the density of 1.5×10^5 cells/well in a 24-well plate followed by transfection with indicated expression vector. After 24 h of transfection, the cells were treated with TNF-α for four hours and fixed with 4% paraformaldehyde. The cells were monitored by Zeiss LSM 710 confocal microscope (Carl Zeiss, Inc., Germany). Images were captured, pseudocolored and analyzed by Zen Black software. Colocalization was quantified by correlation using Image J 1.45 software (NIH, MD, USA).

2.7. Subcellular fractionation and Western blotting

Mitochondrial localization of NLRX1 and Caspase-8 were analyzed by Western blotting using isolated mitochondria. HEK293 cells were plated at the density of 2×10^6 cells/90 mm^2 dish and transfected with indicated constructs using calcium phosphate transfection method. Cells were treated with TNF-α/CHX for 8 h and washed with cold PBS, harvested by centrifugation at 600 ×*g* for 10 min. Cell pellets were resuspended in mitochondria isolation buffer (200 mM mannitol, 70 mM sucrose, 1 mM EGTA, 10 mM HEPES; pH 7.4, 1× protease inhibitor cocktail (Sigma-Aldrich, USA) and incubated on ice for 5 min. The cells were disrupted by passing through a 24G sterile syringe needle and centrifuged at 600 ×*g* for 10 min to separate nuclei and cell debris. The supernatant (cytosolic fraction) was collected and centrifuged again at 10,000 ×*g* for 10 min. The pellet (mitochondrial fraction) were washed twice with isolation buffer and lysed with mitochondria lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.2% Triton X-100, 0.3% NP40 and 1× Protease Inhibitor Cocktail. The mitochondrial and cytosolic protein concentrations were assessed using Bradford protein estimation assay. Equal proteins were loaded and resolved on 12% SDS-PAGE and analyzed by Western blotting using indicated antibodies.

2.8. ROS measurements

Intracellular and mitochondrial ROS production was measured by CM-H_2DCFDA (10 μM) and MitoSOX Red (5 μM) staining respectively. Briefly, HEK293 and HeLa cells were plated in 24-well plates at the density of 1.5×10^5 cells/well. After overnight incubation, the cells were transfected with NLRX1 and NLRX1 shRNA1 using calcium phosphate transfection protocol. Twenty-four hours post transfection, the cells were treated with indicated reagents for 4 h and stained with indicated reagent and monitored under fluorescence microscope (Olympus IX81 microscope; Olympus, Tokyo, Japan). Minimum of 5 images and 80–100 cells were used for analysis.

Similarly, ROS levels were quantified by fluorometry. Briefly, MCF-7 and HEK293 cells were transfected with indicated constructs. The cells were treated with indicated reagents for 4 h and stained with CM-H_2DCFDA (12.5 μM) in DPBS for intracellular ROS quantification and MitoSOX Red (2.5 μM) in DMEM for mitochondrial superoxide quantification. The cells were washed with DPBS and normalized to1×10^6 cells/ml. Fluorescence intensity was quantified by fluorometer (Hitachi High-Technologies Corp., Japan) with excitation/emission at 495/520–540 nm and 510/570–600 nm, respectively.

2.9. Mitochondrial Complex I and Complex III assays

The activities of mitochondrial Complex I and Complex III was analyzed spectrophotometrically. Briefly, HEK293 cells were seeded at the density of 5×10^5 cells/well in the 6-well plate. After overnight incubation, the cells were transfected with indicated construct using calcium phosphate transfection protocol and treated as indicated. The NLRX1 knockdown stable cell line and the NLRX1 expressing cells were harvested and washed with cold DPBS. The cells were subjected to 2–3 freeze–thaw cycles in a freeze–thaw complete solution (0.25 M sucrose, 20 mM Tris–HCl (pH 7.4), 40 mM KCl, 2 mM EDTA supplemented with 1 mg/ml fatty acid-free BSA, 0.01% Digitonin and 10% Percoll). The cells were washed again with the freeze–thaw solution devoid of digitonin and resuspended in Complex I assay buffer (35 mM potassium phosphate (pH 7.4), 1 mM EDTA. 2.5 mM NaN_3,1 mg/ml BSA, 2 μg/ml antimycin A, 5 mM NADH). The reaction was started by adding 80 μg of cell lysate to 500 μl of assay buffer in 1 ml quartz cuvette. Complex I activity was measured for 3 min by monitoring the decrease in absorbance at 340 nm after the addition of 2.5 mM acceptor decylubiquinone indicating the oxidation of NADH.

Similarly, for Complex III activity, NLRX1 knockdown stable cells were seeded at a density of 2.5×10^6 cells/90 mm^2 dish. The cells were harvested and washed with cold DPBS. All the subsequent steps were performed at 4 °C. The cells were suspended in 0.5 ml of 20 mM hypotonic potassium phosphate buffer (pH 7.5) and lysed using a 24G sterile syringe and subjected to freeze–thaw cycle. The cell lysate (80 μg) was added to the 500 μl of Complex III assay buffer (25 mM potassium phosphate (pH 7.5), 0.025% Tween-20, and 300 μM NaN_3, 75 μM Cytochrome c) in cuvette and the baseline observance was monitored at 550 nm for 2 min. The reaction was started by the addition of 100 μM decylubiquinol (freshly prepared 2.5 mM stock) monitoring the increase in absorbance at 550 nm for 2 min.

2.10. ATP measurements

The levels of ATP were measured in NLRX1 expressing and NLRX1 KD cells under different conditions by an ATP dependent luciferase assay using ATP determination kit (Molecular Probes/Life Technologies, ON, Canada).

2.11. Soft agar assay, colony formation assay and scratch assay

Anchorage-independent potential was assessed by soft agar assay, clonogenic activity and migration ability of cancer cells were determined as described previously [31,36].

2.12. Animal experiments

Animal studies were performed according to the rules and protocols approved by the Bioethical committee of the Engelhardt Institute of Molecular Biology, Moscow, Russia. A total of 48 nude mice (5 week old Balb/c *nu/nu*) were used in the current expeiment. RKO colon carcinoma cells carrying empty lentiviral vector pLSLP, NLRX1 shRNA1 (Sigma TRCN0000129459) and over expressing NLRX1 (lentiviral construct pLCMV-NLRX1-puro) were used. Cells were trypsinized, washed three times in ice-cold PBS and inoculated subcutaneously in four locations (left and right hind and shoulders) at a density of 1×10^6 cells in 0.1 ml per inoculum. Fifteen micrograms of purified human recombinant TNF-α (gift of Dr. Alexey Sazykin, Moscow State University) was inoculated intraperitoneally 24 h later, and control mice were inoculated with PBS. The mice were inspected every two days and tumor size was measured. Tumor volume was calculated according to the following formula: ellipsoid volume: $1/6 * pi * a * b * c$, where a, b, and c are linear sizes of the tumor in three dimensions.

Expression levels of NLRX1 transcripts in the cell lines were monitored by real time PCR with EVA-green dye.

The following primers were used:

NLRX1-For: AACGGTGCTGGTGACACA;
NLRX1-Rev: GCTCAGCTCATTGAAGTAGA.

The level of NLRX1 inhibition was 85% in RKO cells. Levels of NLRX1 transcripts in the hyperexpressor cell lines were 8-fold compared to

controls for RKO cells. Basal levels of NLRX1 transcripts (compared to β-actin control transcripts) were roughly similar for RKO cells.

2.13. Statistical analysis

Data are expressed as mean ± SEM of at least two or three independent experiments. Unpaired two-tailed Student's t tests were performed unless otherwise noted and *P < 0.05, **P < 0.01 and ***P < 0.001 were considered to denote significance. GraphPad Prism® was used to perform all the statistical analyses.

3. Results

3.1. NLRX1 sensitizes TNF-α induced cell death

To study the role of NLRX1 in regulation of cell death and survival in response to different physiological stress, we transfected HEK293 cells with NLRX1, exposed the cells to different stimuli and monitored cell death by trypan blue exclusion assay (Fig. 1A). The expression of NLRX1 had no significant effect on cell survival in the untreated conditions. The expression of NLRX1 showed no effect on cell death in response to oxidative stress induced by Rotenone and H_2O_2. The co-treatment of NLRX1-expressing cells with TNF-α and the translation inhibitor cyclohexamide (CHX) showed significantly increased cell death (Fig. 1A). Similarly, transfection of HeLa cells with NLRX1 significantly increased the number of trypan blue positive cells following the TNF-α/CHX treatment (Fig. S1A). These results suggest that NLRX1 specifically sensitizes the cells to TNF-α induced death. This conclusion was further confirmed by downregulating NLRX1 using specific shRNA in HEK293 cells. Three different shRNAs were checked for the efficient knockdown of NLRX1. The shRNA1 of NLRX1 showed maximum down-regulation hence it was further used in the study (Fig. 1B). The constructs expressing NLRX1-specific shRNAs were transfected to HEK293 cells and cell death was monitored by trypan blue exclusion staining. Knockdown of NLRX1 by all of the shRNAs increased the cell survival following TNF-α/CHX treatment, although significant increase was achieved with shRNA1 (Fig. 1C). Similarly, the transfection of NLRX1 shRNA1 in HEK293 showed maximum cell survival (Fig. S1B). To further

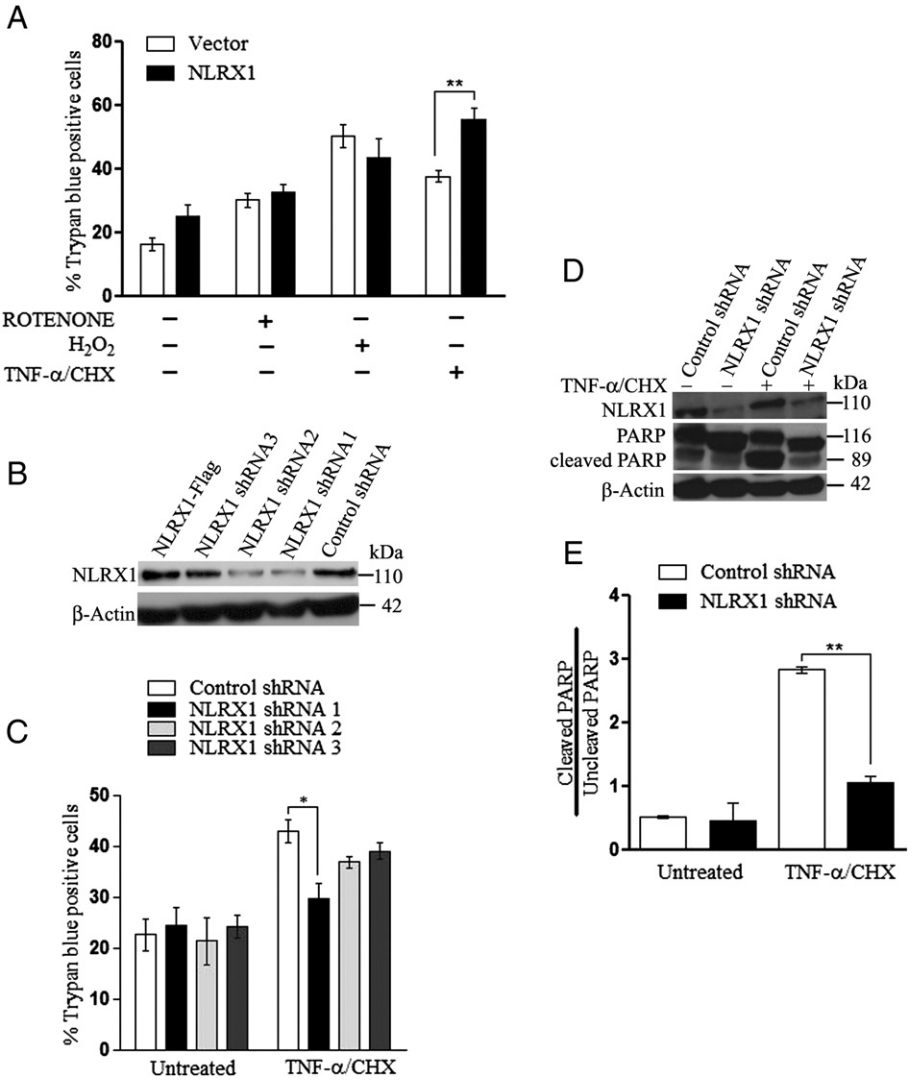

Fig. 1. NLRX1 regulates TNF-α induced cell death. (A) HEK293 cells were transfected with NLRX1 and vector control followed by treatment with Rotenone (50 μM), H_2O_2 (100 μM) and TNF-α/CHX (10 ng/25 μM). The cell death was monitored by Trypan blue exclusion assay. (B) HEK293 cells were transfected with control shRNA, NLRX1-Flag and three different shRNAs targeting NLRX1, namely NLRX1 shRNA 1, 2 and 3. Cell lysates were analyzed for NLRX1 expression by Western blotting. (C) HEK293 cells were transfected with control shRNA and three shRNA targeting NLRX1. Cells were subjected to TNF-α/CHX treatment for 24 h and analyzed cell death by Trypan blue staining. (D) NLRX1 shRNA and control shRNA stable HEK293 cells were treated with TNF-α/CHX for 6 h. Cell lysates were subjected to Western blotting with PARP specific antibody. (E) Quantification of PARP cleavage detected by Western blotting was performed using densitometry and ratios of cleaved to uncleaved PARP are indicated (results are representative of two independent experiments). Data in (A) and (C) depict mean ± SEM values (n = 3). Asterisk (*) indicates that p value < 0.05, for SEM.

investigate the mechanism of TNF-α induced cell death, PARP immunoblotting was performed after NLRX1 knockdown. PARP is cleaved by executioner caspases during TNF-α induced apoptosis [37]. NLRX1 knockdown HEK293 cells were treated with TNF-α/CHX for 6 h and PARP cleavage was monitored. Western blotting showed 110 kDa and 89 kDa band corresponding to native and cleaved form of PARP in control shRNA transfected cells. The knockdown of NLRX1 showed decreased levels of 89 kDa bands corresponding to cleaved subunit of PARP as compared to the control (Fig. 1D, E). To rule out the possibility of a cell line-specific phenomenon, the expression of NLRX1 in MCF-7 cells also showed increased levels of cleaved PARP subunit as compared to vector transfected cells (Fig. S1C). Collectively, the results indicate that NLRX1 is an important regulator of TNF-α induced cell death. The similar study from Soares et al. [38] also reported a key role of NLRX1 in cell death and cancer susceptibility during the preparation of the manuscript. Several findings from our study is in consonance with this study, however, the two studies differ in respect to the response to TNF-α/CHX treatment. It is highly probable that transformed cell lines with a variable expression level of NLRX1 may differ in sensitivity to TNF-α/CHX. The cell death data reported in the current study showed different levels of cell death in HEK293, MCF-7 breast and HeLa which had a different expression level of NLRX1.

3.2. Caspase activation is essential for the NLRX1 mediated sensitization of TNF-α induced cell death

The cleavage of PARP suggests the activation of caspases in the presence of NLRX1. The activation of caspases plays a major role in TNF-α induced cell death [18] hence we planned to confirm the role of caspases in the NLRX1 mediated sensitization to TNF-α induced cell death. Firstly, we analyzed Caspase-8 and Caspase-3 activations at different time points by Western blotting (Fig. 2A). High levels of 41 kDa and 18 kDa bands corresponding to cleaved subunits p43 and p18 of Caspase-8 were detected as early as 4 h in the presence of TNF-α/CHX in NLRX1 transfected HEK293 cells, as compared to vector transfected cells (Fig. 2A). Similarly, a 17 kDa band corresponding to the cleaved subunit of Caspase-3 was detected 4 h after the TNF-α/CHX treatment (Fig. 2A). Quantification of Caspase-8 activity was also monitored at different time points in HEK293 cells transfected with vector control and NLRX1 in the presence of TNF-α/CHX. The enzymatic activity of Caspase-8 was high at 4 h and remained elevated till 8 h in NLRX1 transfected cells as compared to vector control (Fig. 2B). Similarly, the expression of NLRX1 in MCF-7 cells showed increased levels of cleaved Caspase-8 subunits as compared to vector transfected cells in the presence of TNF-α/CHX. (Fig. S1C). Conversely, knockdown of NLRX1 in HEK293 cells showed a significant decrease in the levels of cleaved subunits of Caspase-8 (p43 and p18) as compared to control (Fig. 2C).

To confirm the role of NLRX1 in TNF-α induced Caspase-8 activation and cell death, Caspase-8 activity was monitored upon co-treatment of the cells with the pan-caspase inhibitor, z-VAD-fmk and TNF-α/CHX at the 4 h time point. A decrease in Caspase-8 enzymatic activity was observed following inhibition of caspases in NLRX1 transfected cells (Fig. 2D). This result was confirmed by Western blotting showing reduced levels of cleaved Caspase-8 subunits in the presence of z-VAD-fmk (Fig. 2E). Similarly, HEK293 cells were transfected with NLRX1, treated with TNF-α or with TNF-α/CHX in the absence or presence of the pan-caspase inhibitor z-VAD-fmk and monitored cell death. We observed no significant difference in cell death between NLRX1-transfected and vector-transfected cells, both in untreated conditions and in the presence of TNF-α. Treatment with TNF-α/CHX sensitized NLRX1 expressing cells to TNF-α induced cell death as observed earlier. The co-treatment of the NLRX1 expressing cells with TNF-α/CHX and z-VAD-fmk attenuated cell death (Fig. 2F). Interestingly, the co-treatment of cells with the Caspase-8 specific inhibitor z-IETD-fmk significantly increased cell survival as assessed by trypan blue staining. All the above evidences strongly suggest the role of NLRX1 in regulation of TNF-α-mediated Caspase-8 activation.

3.3. NLRX1 associates with the TNF-α induced complex-II to promote Caspase-8 activity

The binding of TNF-α to its cell surface receptor and formation of complex-II initiates cell death program by recruiting Caspase-8 and other adaptor proteins. The observed stimulation of Caspase-8 activity by NLRX1 as early as 4 h after TNF-α/CHX treatment suggests that NLRX1 may act at the level of complex-II formation. Recent studies implicate an essential role of ubiquitin ligases in the assembly and activation of Caspase-8 [35,39,40]. RING family E3 ligase TRAF2 interacts with Caspase-8 at complex-II and sets a threshold for Caspase-8 activation [39]. Similarly, our recent observation has demonstrated the role of the RING E3 ligase, TRIM13 in regulation of Caspase-8 activation downstream of complex-II [35]. We planned to test the association of NLRX1 with TNF-α induced signaling complexes and the role of the E3 ligases in regulation of NLRX1 mediated activation of Caspase-8. HEK293 cells were co-transfected NLRX1 with TRAF2 and TRIM13 and were treated with TNF-α/CHX. In agreement with our earlier report [35], the expression of TRIM13 increased the levels of 41 kDa and 18 kDa bands corresponding to processed Caspase-8 in the absence/presence of TNF-α/CHX (Fig. 3A). However, the co-expressions of TRIM13 and NLRX1 decreased the activation of Caspase-8 in the presence of TNF-α/CHX. Similarly, in agreement with earlier report the expression of TRAF2 attenuated the processing and activation of Caspase-8 to that of control level. Co-expression of TRAF2 significantly reversed the NLRX1-mediated increase in levels of processed Caspase-8 in presence of TNF-α. Taken together, the data supported our hypothesis that NLRX1 affects the activity of Caspase-8 at the level of complex-II formed after TNF-α stimulation. To test whether NLRX1 may interact with components of complex-II, we performed co-immunoprecipitation (IP) analysis. We co-expressed NLRX1 along with HA-TRAF2 and Myc-RIP1 in HEK293 cells, treated the cells with TNF-α/CHX for 5 min and 8 h to analyze Complexes I and -II, respectively. We immunoprecipitated TRAF2, a subunit of both complexes with HA-antibodies and analyzed the interactions. By Western blotting we identified a 110 kDa band corresponding to NLRX1, a 78 kDa band of RIP1 and Casaspe-8, after eight hours of TNF-α/CHX treatment. There was no pull down of NLRX1, RIP1 and Caspase-8, after five minutes of TNF-α/CHX treatment (Fig. 3B). The result indicates that NLRX1 associates with TNF-α induced signaling complex-II to promote the activation of Caspase-8.

3.4. NLRX1 localizes to mitochondria and augments the TNF-α induced ROS production

NLRX1 localizes to mitochondria, although its functional significance is still not well understood. Similarly, Caspase-8 is also known to be localized to mitochondria hence, we hypothesized that the crosstalk between NLRX1 and Caspase-8 may regulate mitochondrial function in response to TNF-α. We analyzed the cellular localization of NLRX1 in the absence/presence of TNF-α. We transfected MCF-7-MT-RFP stable cell line with NLRX1-GFP and monitored the cellular localization of proteins by confocal microscopy. We observed that a significant fraction of NLRX1 co-localizes with mitochondria (Fig. 4A, B) in the presence of TNF-α. This observation was further confirmed by subcellular fractionation. The mitochondrial and cytoplasmic fractions from the TNF-α/CHX treated NLRX1-HEK293 cells were analyzed by Western blotting. The high level of pro-caspase-8 was detected (the 55 kDa band) in the mitochondrial fraction of untreated cells. The increased levels 43 kDa and 18 kDa corresponding to the cleaved subunits of Caspase-8 (p43 and p18) were higher in NLRX1 transfected mitochondrial fraction as compared to vector control in the presence of TNF-α/CHX. NLRX1 was specifically enriched in the mitochondrial fraction of both untreated and treated cells (Fig. 4C). The quality of mitochondrial and cytosolic

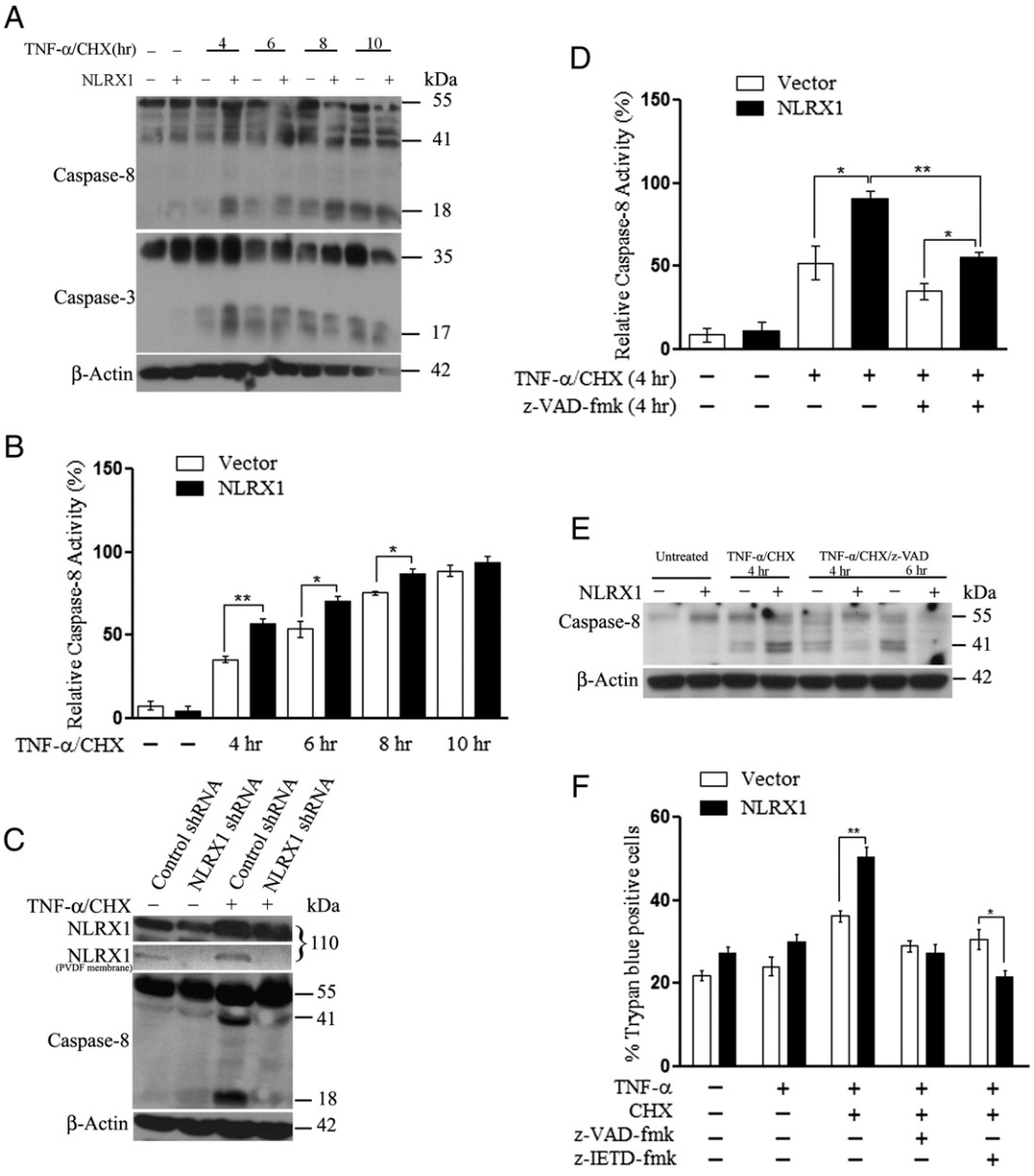

Fig. 2. NLRX1 regulates Caspase-8 activity during TNF-α induced cell death. (A) HEK293 cells were transfected with NLRX1 and treated with TNF-α/CHX for indicated time. The cell lysates were analyzed by Western blotting using Caspase-8 and Caspase-3 specific antibodies. (B) HEK293 cells were transfected and treated as indicated in (A) and Caspase-8 activity was analyzed at different time points by Caspase-8 Glo luciferase assay system (n = 2).(C) NLRX1 shRNA and control shRNA stable HEK293 cells were treated with TNF-α/CHX for 4 h and Caspase-8 activation was analyzed by Western blotting. (D and E) HEK293 cells were transfected with NLRX1 and vector and treated with TNF-α/CHX or TNF-α/CHX and z-VAD-fmk (20 μM) for indicated time. The Caspase-8 activation was analyzed by Caspase-8 Glo luciferase assay system (n = 2) (D) and Western blotting (E). (F) HEK293 cells were transfected with NLRX1 and vector control followed by treatment with TNF-α/CHX, z-VAD-fmk (20 μM) and z-IETD-fmk (10 μM). After 24 h of treatment, cell death was measured by trypan blue exclusion assay (n = 3). Asterisk (*) indicates that p value < 0.05, for SEM.

fractions was also analyzed using antibodies to NDUFS2, a subunit of mitochondrial Complex I, and to RPS9, a component of 40S ribosomal subunit. The NDUFS2 was found exclusively in the mitochondrial fraction whereas RPS9 was detected only in the cytosol. Together, these results demonstrate that NLRX1 localizes to mitochondria in the presence of TNF-α/CHX.

Emerging evidences suggest that caspases may cleave subunits of the ETC and regulate ROS during different stress conditions [41]. The role of the crosstalk between NLRX1 and Caspase-8 in regulation of ROS and activity of mitochondrial complexes of ETC is not well understood. To test whether NLRX1 may regulate TNF-α induced ROS generation, the NLRX1 knockdown HeLa cells were treated with TNF-α/CHX and stained with the oxidant-sensitive dye, CM-H_2DCFDA. As expected, the TNF-α/CHX treatment increased ROS levels in control cells. Interestingly, the knockdown of NLRX1 substantially attenuated the TNF-α/CHX stimulated generation of ROS (Fig. 5A). To confirm this, intracellular ROS formation in HEK293 cells was quantified in the presence of TNF-α/CHX alone or in combination with N-Acetyl Cysteine (NAC), an antioxidant. ROS generation was significantly decreased in NLRX1 knockdown (KD) HEK293 cells while co-treatment with NAC further suppressed ROS level in NLRX1 KD HEK293 cells in the presence of TNF-α/CHX (Fig. 5B). Based on these results, we concluded that NLRX1 localizes to mitochondria and participates in the TNF-α induced ROS generation.

Mitochondria is one of the major sites of ROS under different stress conditions. We hypothesized that a cross talk between NLRX1 and Caspase-8 may regulate mitochondrial function, including the generation of ROS. To test this possibility, mitochondrial superoxide generation in NLRX1 and vector transfected MCF-7 cells was quantified in the presence of TNF-α/CHX alone or in combination with Mito-TEMPO, a

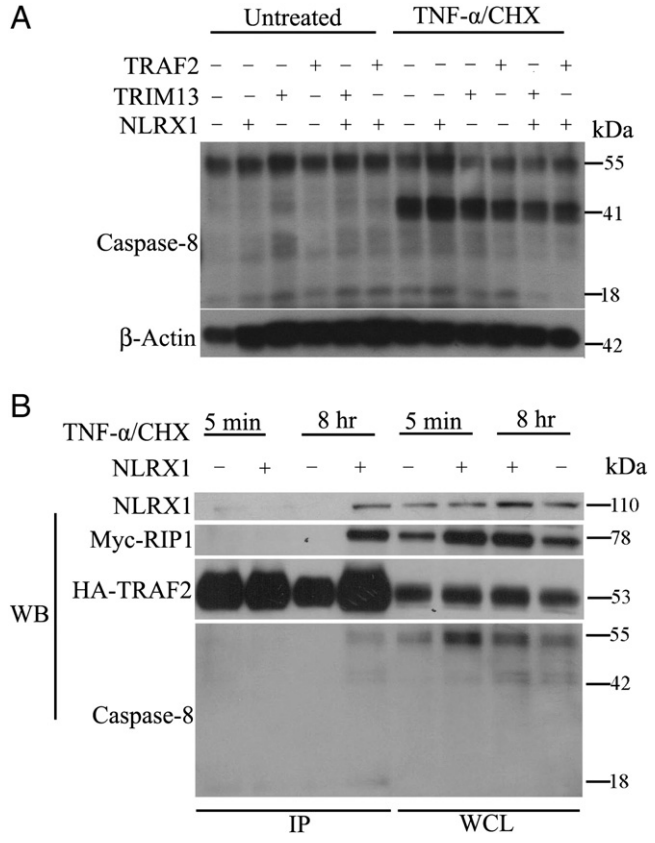

Fig. 3. NLRX1 interacts with complex-II during TNF-α induced cell death. (A) HEK293 cells were cotransfected with NLRX1 in combination with TRAF2 and TRIM13. The cells were treated with TNF-α/CHX for 4 h. Cell lysates were analyzed by Western blotting with Caspase-8 antibody. (B) HEK293 cells were transfected with NLRX1-Flag, HA-TRAF2, Myc-RIP1 followed by co-treatment with TNF-α/CHX for 5 min and 8 h. Immunoprecipitation with anti-HA antibody was performed and interacting proteins were analyzed by Western blotting with indicated antibodies.

mitochondrial superoxide scavenger. The expression of NLRX1 significantly induced mitochondrial ROS in the presence of TNF-α/CHX while co-treatment with Mito-TEMPO suppressed superoxide formation as compared to vector control (Fig. 5C). Similarly, we treated NLRX1 transfected HEK293 cells with TNF-α/CHX and mitochondrial ROS level was analyzed by staining cells with MitoSOX Red and visualized under fluorescence microscope. NLRX1 expression alone stimulated mitochondrial superoxide formation in untreated condition which further increased significantly in the presence of TNF-α/CHX as compared to vector control (Fig. S2A).

To determine the role of Caspase-8 in amplification of TNF-α induced ROS generation, HeLa cells expressing NLRX1 were pretreated with z-IETD-fmk, then treated with TNF-α/CHX and the intracellular ROS was assessed by CM-H_2DCFDA staining. ROS levels were significantly increased in NLRX1 transfected cells as compared to vector transfected cells in the presence of TNF-α/CHX. The inhibition of Caspase-8 with z-IETD-fmk decreased the level of ROS in NLRX1 transfected cells treated with TNF-α/CHX (Fig. 5D). ROS quantification in MCF-7 cells transfected with NLRX1 and vector control yielded similar results both in the presence of TNF-α/CHX and co-treatment with z-IETD-fmk (Fig. 5E). We also tested whether blocking ROS generation may rescue TNF-α/CHX induced death of NLRX1 expressing MCF-7 and HEK293 cells. As observed earlier, expression of NLRX1 had no significant effect on cell survival in the untreated conditions and in the presence of Rotenone and H_2O_2. Treatment with TNF-α/CHX sensitized NLRX1 expressing cells to TNF-α induced cell death, in contrast, co-treatment with NAC significantly increased cell survival of NLRX1 transfected cells as compared to vector transfected cells (Fig. S2B). Similar results were obtained in HEK293 cells transfected with NLRX1 and treated with TNF-α/CHX along with Mito-TEMPO (Fig. S2C). All together, these data suggested that NLRX1 regulates Caspase-8 activity to modulate mitochondrial ROS and cell death during TNF-α induced apoptosis.

3.5. NLRX1 regulates mitochondrial respiratory Complex I and Complex III to maintain ATP levels in the presence of TNF-α

As Complex I and Complex III of the mitochondrial electron transport chain are the major sites for ROS generation [42], hence we tested if NLRX1 expression may regulate their activity. The expression of NLRX1 in HEK293 cells decreased Complex I activity in the presence of TNF-α, but not after co-treatment with TNF-α and CHX. Interestingly, inhibition of Caspase-8 by z-IETD-fmk significantly increased Complex-I activity in the presence of TNF-α/CHX (Fig. 6A). Similarly, the treatment of cells with pan-caspase inhibitor z-VAD-fmk enhanced Complex I activity to the same level in both NLRX1 expressing and control cells. Conversely, the activity of respiratory Complex I increased, both in the absence/presence of TNF-α in shNLRX1 transfected cells (Fig. 6B). Similarly, the knockdown of NLRX1 in HEK293 cells increased Complex III activity, as compared to control cells, both in the absence and presence of TNF-α (Fig. 6C).

The above experiments suggest that NLRX1 regulates the activity of mitochondrial complexes which is the major source of ATP generation in the cell. Therefore, we investigated whether the expression of NLRX1 might regulate total cellular and mitochondrial ATP levels in the presence of TNF-α. The expression of NLRX1 in HEK293 cells decreased total cellular steady-state ATP level in the presence of TNF-α whereas the decrease was less evident when the cells were co-treated with TNF-α and CHX (Fig. 6D). The ATP levels were measured after 16 h of treatment with TNF-α/CHX, hence, the activated caspases may have cleaved the subunits of ETC complexes as observed earlier [23] and the decrease may not be evident. Importantly, the inhibition of Caspase-8 by z-IETD-fmk during TNF-α/CHX induced apoptosis significantly enhanced the ATP levels in NLRX1 expressing cells, while addition of z-VAD-fmk showed no significant effect on the levels of ATP. The inhibition may not be observed as z-VAD-fmk is not a potent inhibitor of the initiator caspases [43] further suggesting that activated caspase-8 may directly act on complexes of ETC in the presence of NLRX1. Total ATP levels in the NLRX1 KD HEK293 cells were significantly higher in both TNF-α and TNF-α/CHX treated cells (Fig. 6E). The mitochondrial ATP levels in NLRX1 KD HEK293 cells increased both in the absence/presence of TNF-α (Fig. S2D). Similar results were observed in MCF-7 breast carcinoma cells where NLRX1 expression showed a significant decrease in total ATP level both in the absence/presence of TNF-α (Fig. S2E). These results strongly suggest that NLRX1 regulates activity of mitochondrial complexes and maintenance of intracellular levels of ATP.

3.6. NLRX1 expression suppresses clonogenic ability, anchorage-independent growth and migration of cancer cells in vitro

The NLRX1 mediated decrease in mitochondrial respiratory complex activity suggests its important role in cancer cell metabolism. Extracellular acidification of tumor microenvironment is a common feature of tumor tissue. The acidic pH is attributed to a switch from mitochondrial respiration to aerobic glycolysis, which is a characteristic hallmark of cancer cells [30]. Interestingly, we observed that knockdown of NLRX1 in HeLa cells reversed the acidification of the culture medium (Fig. S2F), suggesting that NLRX1 may contribute to the metabolic switch toward glycolysis in tumor cells.

As we observed above that NLRX1 can modify mitochondrial metabolism and promote aerobic glycolysis, it may have an implication in regulating the clonogenic and tumorigenic potentials of tumor cells. We analyzed the NLRX1 expression in different cancer types available in

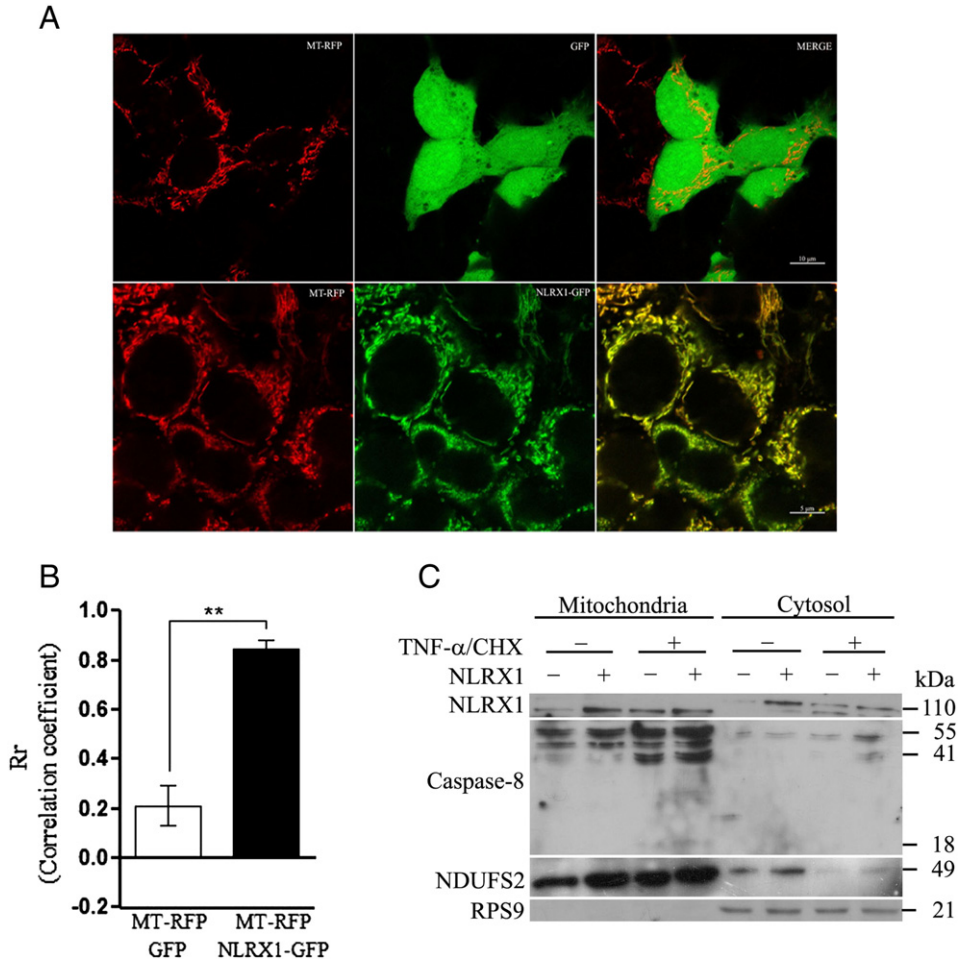

Fig. 4. NLRX1 translocates to mitochondria and regulates Caspase-8 activity at the mitochondria in the presence of TNF-α. (A) MCF-7-MT-RFP stable cell line was transfected with NLRX1-GFP and GFP control. After 24 h of transfection, cells were treated with TNF-α, fixed with 4% paraformaldehyde and visualized by confocal microscopy. Scale bar in the upper and lower panel represents 10 μm and 5 μm, respectively. (B) Colocalization quantification of confocal images by Pearson's correlation coefficient (Rr) was performed using Image J 1.45 software (NIH, MD, USA). (C) HEK293 cells were transfected with NLRX1 and control vector followed by treatment with TNF-α/CHX for 4 h. Cells were subjected to subcellular fractionation. Mitochondrial and cytoplasmic fractions from untreated and treated cells were analyzed by Western blotting with indicated antibodies. Asterisk (*) indicates that p value < 0.05, for SEM.

gene expression databases. NLRX1 mRNA levels were downregulated in breast and cervical cancers (Gene Expression across Normal and Tumor tissues (GENT), Fig. S3A, (http://medicalgenome.kribb.re.kr/GENT)). Similarly, protein expression levels of NLRX1 were lower in breast tissues as compared to normal breast tissues (Fig. S3B) (http://www.proteinatlas.org/). Therefore, we examined NLRX1 expression pattern in different breast cancer cell lines using Western blotting. NLRX1 protein levels were low in MCF-7 and T47D (ER positive) cells as compared to MDA-MB-231 and HBL-100 (ER negative) cells that demonstrated elevated levels of NLRX1 (Fig. 7A). We investigated whether NLRX1 expression in MCF-7 affected cancer phenotype using clonogenic assay and soft agar tumorigenesis assay. The clonogenic ability of NLRX1 expressing MCF-7 cells was monitored in the presence and absence of TNF-α. The expression of NLRX1 significantly decreased the clonogenic ability of MCF-7 cells in the presence of TNF-α (Fig. 7B) also shown as plating efficiency (Fig. 7B′). The soft agar assay was performed to study the role of NLRX1 in anchorage-independent cell growth. Control MCF-7 cells were able to grow in soft agar and formed large colonies in the presence of TNF-α. The NLRX1 expressing MCF-7 cells were not able to grow in soft agar and formed small colonies in the presence of TNF-α, as compared to untreated cells (Fig. 7C) plotted as colony area (Fig. C′). We further checked whether NLRX1 expression may inhibit cell migration. MCF-7 cells were transfected with NLRX1, treated with TNF-α treatment and the migration ability was analyzed by scratch assay. The NLRX1 expressing MCF-7 cells displayed a significant increase in open wound area in the presence of TNF-α, as compared to vector transfected cells (Fig. 7D). The low level of expression of NLRX1 in MCF-7 may still regulate the cellular functions hence we further downregulated the expression by shRNA transfection. The NLRX1 knockdown in MCF-7 cells displayed an increased ability to form colonies (tumorispheres) in soft agar, as compared to control cells. The size of colonies was also increased significantly in the presence of TNF-α (Fig. S4A and S4B). Collectively, these results suggest that NLRX1 negatively regulates tumorigenic potential and migration ability in untreated condition as well as in the presence of TNF-α.

3.7. NLRX1 suppresses tumorigenicity in nude mice

To extend the cellular finding to in vivo models, we used RKO colon carcinoma cells to perform xenograft tumor formation assays in nude mice. RKO cells expressing NLRX1 shRNA1 or ectopic full length NLRX1 mRNA were injected into nude mice and the growths of subcutaneous tumors were examined every two days. In agreement with our in vitro studies, NLRX1 hyper expression negatively affected the tumorigenic potential of RKO in nude mice. In contrast, NLRX1 knockdown substantially increased the tumorigenic potential of RKO cells in the nude mice xenograft assay (Fig. 8A). Downregulation of NLRX1 significantly increased tumor volume, as well as the increased efficiency of tumor initiation. Similarly, hyper expression of NLRX1 resulted in smaller tumor volume. The effects were

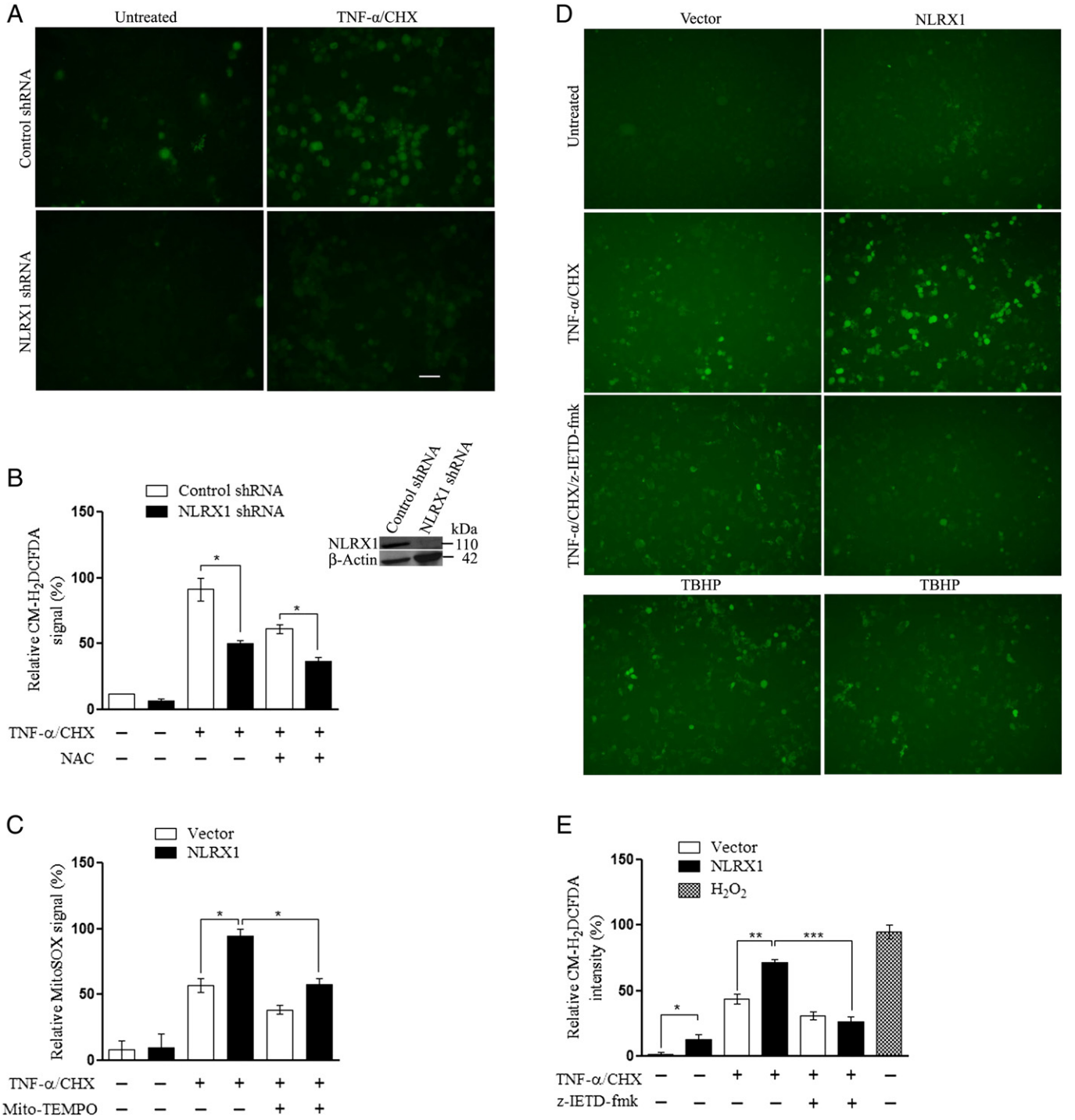

Fig. 5. NLRX1 regulated Caspase-8 activity modulates the TNF-α induced mitochondrial ROS. (A) NLRX1 shRNA and control shRNA stable HeLa cells were treated with TNF-α/CHX for 4 h. ROS production was monitored by staining the cells with CM-H_2DCFDA fluorescent probe and visualized under fluorescence microscope. Scale bar represents 10 μm (n = 2). NLRX1 knockdown was confirmed by Western blotting using anti-NLRX1 antibody. (B) ROS levels were analyzed in control shRNA and NLRX1 shRNA stable HEK293 cells treated with TNF-α/CHX in the absence/presence of NAC (5 mM) using CM-H_2DCFDA (n = 3). (C) Mitochondrial superoxide levels were quantified in NLRX1 and vector transfected MCF-7 cells treated with TNF-α/CHX in the absence/presence of Mito-TEMPO (10 μM) as described in the Materials and methods section (n = 3). (D) HeLa cells were transfected with NLRX1 and control vector followed by treatment with TNF-α/CHX alone or in combination with z-IETD-fmk for 4 h. The cells were stained with CM-H_2DCFDA and observed ROS production under fluorescence microscope (n = 2). (E) Intracellular ROS levels were quantified in NLRX1 and vector transfected MCF-7 cells following treatment with TNF-α/CHX and z-IETD-fmk either alone or in combination. H_2O_2 treatment (50 μM) was used as a positive control (n = 3). Data in (A)–(E) depict mean ± SEM values. Asterisk (*) indicates that p value < 0.05, for SEM.

significantly altered if mice received intraperitoneal injections of TNF-α, after a subcutaneous inoculation of RKO cells. The TNF-α treatment decreased the tumorigenic potential in all three RKO sublines producing the smallest average tumor volume in NLRX1 expressing cancer cells (Fig. 8B, C). In vivo data from nude mice with NLRX1 hyper expression and NLRX1 knockdown in RKO cells correlate with in vitro data from MCF-7 cell line both in untreated and TNF-α treated condition. However, data with control mice in TNF-α treated condition does not comply with the in vitro study. This could be due to the difference in cell lines used for the in vivo study as both the cells differ in origin and have different tumorigenic capability [44]. Similar results were observed in other transformed

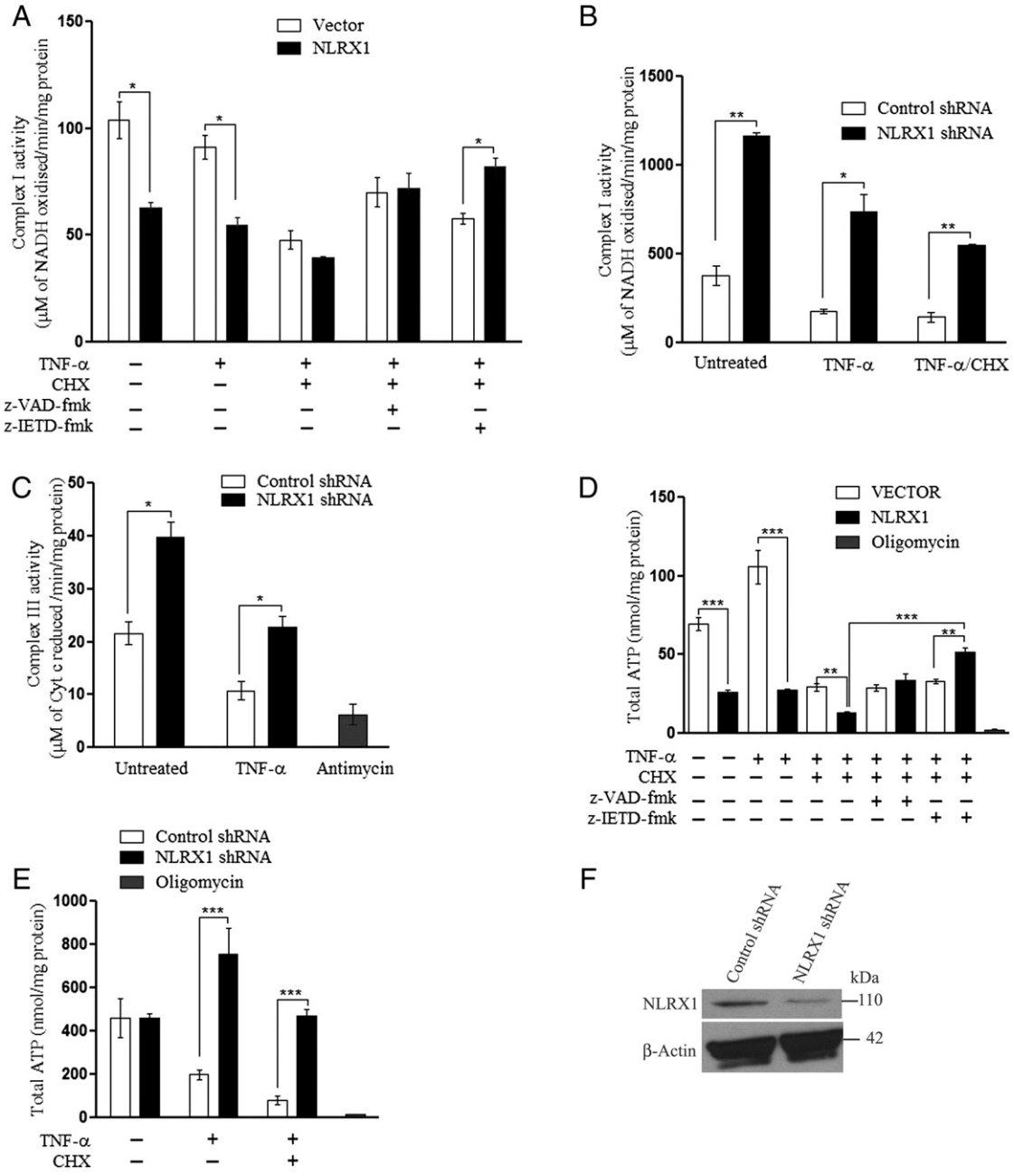

Fig. 6. NLRX1 regulates Complex I and Complex III activity to maintain ATP levels in the presence of TNF-α. (A) HEK293 cells were transfected with NLRX1 and vector control followed by treatment with TNF-α, TNF-α/CHX, z-VAD-fmk and z-IETD-fmk either alone or in combination. (B) and (C) NLRX1 shRNA and control shRNA stable HEK293 cells were treated with TNF-α and TNF-α/CHX. Complex I (B) (n = 3) and Complex III (C) (n = 2) activity were measured spectrophotometrically. (D) HEK293 cells were transfected with NLRX1 and vector control followed by treatment with TNF-α, TNF-α/CHX, z-VAD-fmk and z-IETD-fmk either alone or in combination (n = 3). (E) NLRX1 shRNA and control shRNA stable HEK293 cells were treated with TNF-α and CHX either alone or in combination. ATP levels were measured by ATP-dependent luciferase activity (n = 3). (F) Cell lysates of NLRX1 shRNA stable HeLa cells were analyzed by Western blotting to check NLRX1 expression levels. Data in (A)–(E) depict mean ± SEM values. Asterisk (*) indicates that p value < 0.05, for SEM.

cells expressing NLRX1 and NLRX1 shRNA1 both in the presence and absence of TNF-α (data not shown). These observations suggest that NLRX1 may act as a potential tumor suppressor that abrogates the tumorigenic capability of cancer cells.

4. Discussion

Chronic inflammation is intricately associated with promotion of tumor initiation and progression. The high levels of pro-inflammatory cytokines reprogram metabolic pathways and provide survival advantage to tumor cells [45]. Increased levels of TNF-α have been consistently observed in different tumor types including breast, colorectal and gastric cancers [5]. The role of mitochondria in regulation of inflammatory pathways is emerging. The mitochondria localized NLRX1 has been reported to regulate both IFN and NF-κB pathways [32,33,46]. The role of NLRX1 in controlling inflammation, cell death and metabolism, as well as its participation in cancer initiation and progression is not well understood. In the current study, we demonstrate that NLRX1 sensitizes cells to TNF-α induced death by activating Caspase-8. The crosstalk between NLRX1 and Caspase-8 is important for regulation of mitochondrial ROS and metabolism in tumor cells.

The results obtained in the current study demonstrate that NLRX1 specifically sensitizes the cells to TNF-α induced death but does not affect responses to other stress conditions. TNF-α binds to its cognate

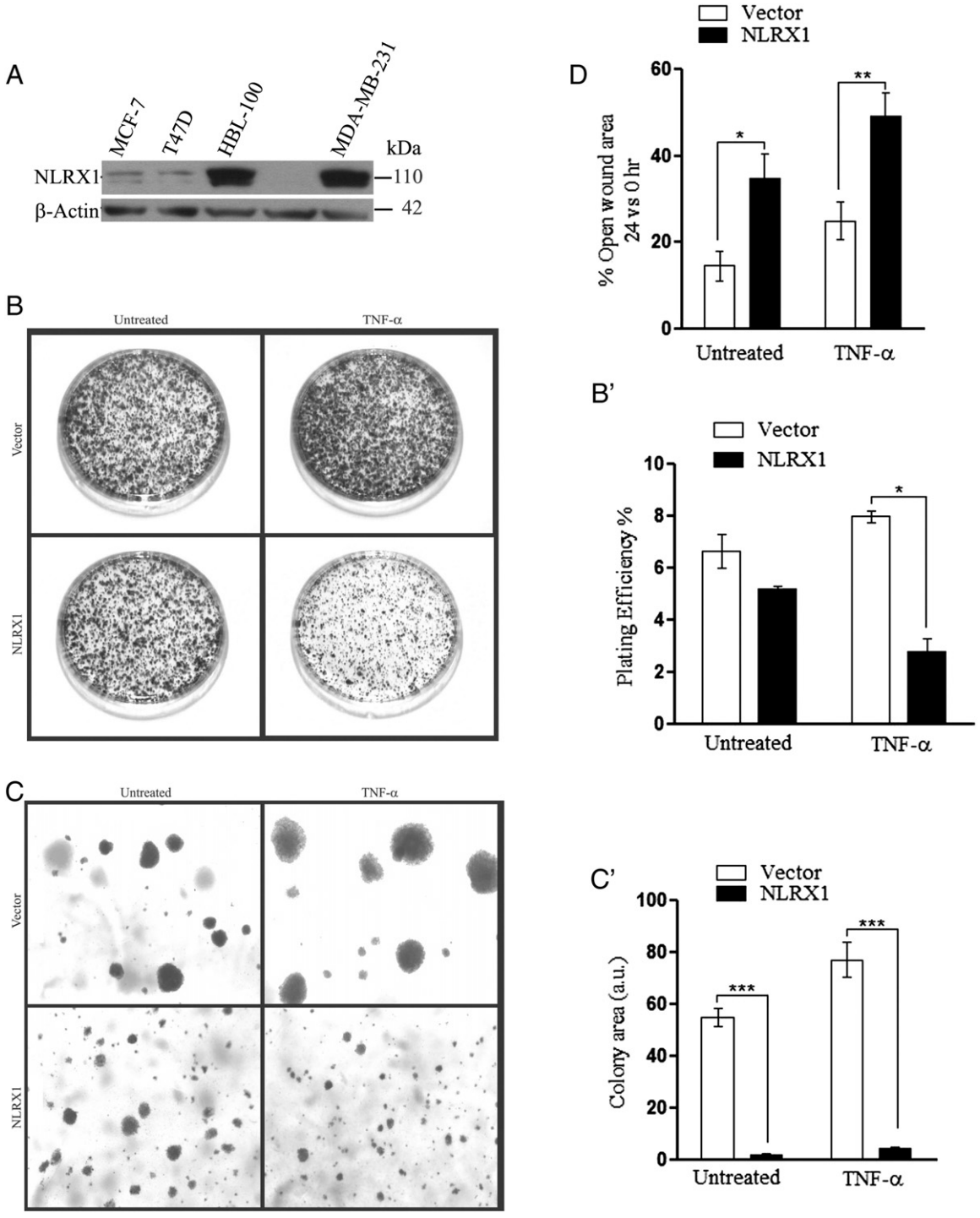

Fig. 7. NLRX1 expression in MCF-7 cells decreases tumorigenic and cell migration property. (A) The expression of NLRX1 was analyzed by Western blotting in MCF-7, T47D, HBL-100 and MDA-MB-231. (B and B') MCF-7 cells were transfected with NLRX1 and control vector followed by treatment with TNF-α and clonogenic activity was assessed by counting number of colony forming units and plotted as plating efficiency (n = 2). (C and C') MCF-7 cells were transfected with NLRX1 and vector constructs and treated with TNF-α. The soft agar assay was performed and colonies in soft agar were observed under DIC mode in fluorescence microscope after 25 days. The colonies areas were measured using Image J 1.45 software (n = 2). (D) MCF-7 cells, transfected with NLRX1 and control vector were subjected to TNF-α treatment and scratch assay was performed as indicated in the Materials and methods section (n = 2). Data in (B)–(D) depict mean ± SEM. Asterisk (*) indicates that p value < 0.05, for SEM.

receptor TNFR1, predominantly expressed on plasma membranes of many different cell types. Following the binding of TNF-α, a proximal membrane bound complex-I is assembled by the recruitment of TRADD adaptor protein, TRAF2, RIP1 and cIAPs. The complex-I is known to activate NF-κB and promotes cell survival. During TNF-α induced apoptosis, complex-I is internalized and recruits Caspase-8 and FADD adaptor protein thereby forming a pro-apoptotic complex-II in the cytoplasm [18]. The exact composition and regulation of the assembly of complex-II during the TNF-α induced apoptosis is not well understood. The results in the current study obtained by measuring cell death and immunoprecipitation demonstrate the association of NLRX1 with Caspase-8 and TRAF2, subunits of complex-II, in the presence of TNF-α. The recruitment of different Ub E3 ligases [35,39,40] at this step may ubiquitinate NLRX1 [33] and may regulate the assembly of the

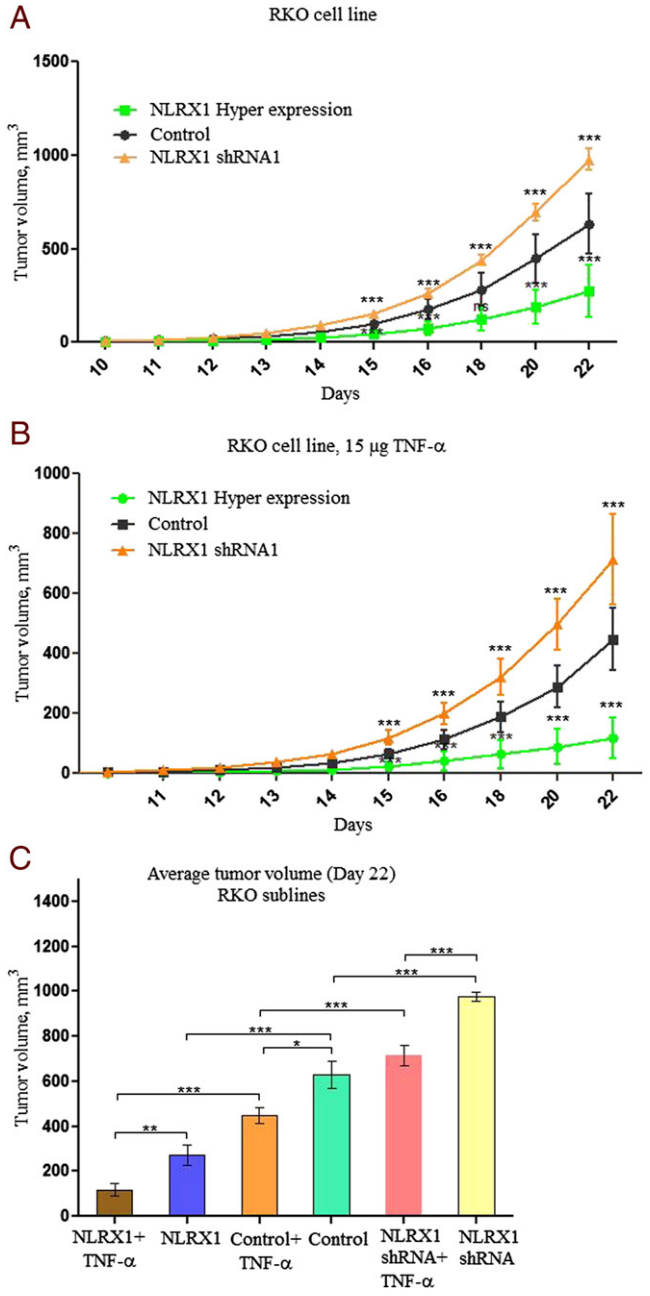

Fig. 8. NLRX1 expression decreases tumorigenic potential of transformed cells in vivo. RKO colon carcinoma cells with stable hyper expression and knockdown of NLRX1 gene were used to study xenograft tumor formation in nude mice. (A) and (B) kinetics of tumor growth in nude mice after injection of RKO cells with hyper expression and stable knockdown of NLRX1 with or without treatment of TNF-α (15 μg/ml) 24 h after administration of cancer cell line. (C) Tumor volume from untreated and TNF-α treated mice was measured every two days. Average tumor volume at day 22 was measured and plotted against each RKO sublines. Data in (A)–(C) depict mean ± SEM of values (n = 3). Asterisk (*) indicates that p value < 0.05, for SEM.

complex and Caspase-8 activation, hence sensitizing the cells to the TNF-α induced cell death.

Caspase-8 is a multifunctional protease and translocates to different subcellular locations during stress conditions. During ischemia conditions, Caspase-8 translocates to the nucleus and cleaves PARP2 [20]. Previous reports also describe the translocation of Caspase-8, TRAF2 and RIP1 to mitochondria during the TNF-α induced apoptosis. Evidences obtained in the current study suggest that NLRX1 may regulate the association of active subunits of Caspase-8 with mitochondria in the presence of TNF-α. NLRX1 may regulate the formation of pro-apoptotic complex-II and mediate activation of Caspase-8 at the mitochondria to control metabolic functions in the presence of TNF-α. This observation is in consonance with earlier report showing that Caspase-8 may form a native macromolecular complex with Bid at the mitochondria [47], although the functional significance of the association was not understood. Our results suggest that NLRX1 and Caspase-8 crosstalk is important for the regulation of mitochondrial ROS, as the inhibition of Caspase-8 and knockdown of NLRX1 attenuate the TNF-α induced mitochondrial ROS. The dysregulation of redox balance in response to TNF-α or certain pathological conditions result in leakage of electrons from Complex I and Complex III to molecular oxygen, thus serving as a primary source of superoxide anion radical. The observed mitochondria-localized association of NLRX1 with activated Caspase-8 suggests that the activity of Caspase-8 may inhibit the mitochondrial ETC Complex I and Complex III. A previous report also suggests that NDUFS1, a 75 kDa protein of mitochondrial Complex I subunit is a substrate of Caspase-3 during apoptosis and is critical event of mitochondrial dysfunction during apoptosis, [41] suggesting caspase mediated regulation of ETC during stress conditions. Similarly, a recent report suggests that Caspase-8 is an important bioenergetic determinant and a key regulator of cellular ATP levels [48]. The results obtained here also allow to hypothesize that NLRX1 may regulate the Caspase-8 activity on mitochondria in the presence of TNF-α. The activated Caspase-8 associated with mitochondria may cleave subunit of mitochondrial respiratory Complex I or Complex III. The association of NLRX1 with UQCRC2, an integral member of the Complex III also known as the mitochondrial cytochrome bc1 complex, is consistent with the hypothesis [49]. The observed TNF-α mediated decrease in the activity of Complex III along with the increase in mitochondria-generated ROS in the NLRX1 expressing cells suggest that activated Caspase-8 may target components of respiratory Complex III. The additional studies are required to understand the association of NLRX1 and Caspase-8 with mitochondrial ETC complexes and its role in metabolism.

Cancer cells are often endowed with high intrinsic ROS generation and constitutive NF-κB activation. Increased ROS generation coincides with upregulated glucose metabolism under hypoxic conditions, a phenomenon characteristic of fast proliferating cancer cells [50,51]. The increased activities of mitochondrial Complex I and Complex III and elevated ATP levels observed in the NLRX1 knockdown cells treated with TNF-α suggest an increase in mitochondrial metabolic activity and energy production. This assumption is supported by a previous report showing that TNF-α regulates the expression of key metabolic enzymes, such as glycogen phosphorylase (PYGL) and glutamate dehydrogenase 1 (GLUD1). PYGL increases the flux of glucose from glycogen reserves whereas GLUD1 activity provides the substrates for anaplerotic metabolism [52]. The current study demonstrates that NLRX1 expression inhibits the activity of mitochondrial respiratory chain, generates ROS and sensitizes cells to TNF-α induced death. Altogether, these results implicate that NLRX1 has a tumor suppressor activity. In breast carcinoma cells, MCF-7 and T47D, the expression of NLRX1 is compromised. We show that the ectopic expression of NLRX1 in MCF-7 cells compromised the clonogenic ability in vitro. In other human carcinoma cell lines RKO, we observed that expression of NLRX1 also decreases clonogenic ability in vitro and tumor formation in a nude mice xenograft assay in vivo. Increased levels of TNF-α observed in tumor microenvironment and loss of NLRX1 expression may facilitate the metabolic reprogramming to meet the anaplerotic demands of tumor cells. The higher expression of NLRX1 was observed in MDA-MB-231, a highly metastatic cell line and HBL-100, a SV40 mediated immortalized cell line. The observations in the current study are supported by the findings of Soares et al. [38]. The authors analyzed WT versus KO cells, rather than scramble versus knockdown cells and found a phenotype between WT and KO cells only in transformed cells that already express very low levels of NLRX1 in WT, rather than in WT versus KO primary

cells that have higher levels of NLRX1 in WTs. It is possible that low expression of NLRX1 in KD cells changes the overall outcome of the sensitivity to TNF-α/CHX.

This strongly suggests that NLRX1 may have a different role in the altered tumor microenvironment. Recent evidence suggests that aggressive and metastatic properties of MDA-MB-231 cells are attributed to autophagy-dependence. These cells are addicted to autophagy for survival even in nutrient rich conditions [53]. Similarly, previous reports suggest that NLRX1 acts as a positive regulator of autophagy during antiviral signaling [54]. Altogether, these evidences raise the possibility that the up-regulated expression of NLRX1 may synergistically regulate metabolism and autophagy for highly invasive growth of the autophagy addicted MDA-MB-231 breast cancer cells. Therefore, the tumor suppressor role of NLRX1 may be cell type dependent and will be highly specific to tumor microenvironment. The heterogeneity observed in the breast cancer cells in tumor microenvironment further strengthens the hypothesis [55]. The responsiveness of these cell lines to TNF-α induced cell death as well as metabolic reprogramming in different cancer types need additional studies.

5. Conclusion

The results obtained in the present study suggest the role of NLRX1 in regulating the cross talk of inflammation, metabolism and tumorigenesis. The proteins discovered at the interface of mitochondria and ER, such as MAVS, MITA and NLRX1, had recently been shown to play important roles in innate immune responses. A previous report from our lab as well as the current study suggest that proteins regulating NF-κB and IFN pathways may be critically linked to metabolism and cell death [56]. The evidences presented here suggest that loss of NLRX1 may confer dual advantage for the cells in the tumor microenvironment, as they acquire resistance to TNF-α induced cell death, while their metabolic pathways are being reprogrammed to meet the high energy and anaplerotic demands. Further studies in this direction would help to understand the intimate connection between inflammation, metabolism and tumor progression.

Acknowledgments

The current research work was financially supported by the Department of Science and Technology (DST), Government of India, Indo-Russia grant number INT/RFBR/P-91 to Dr. Rajesh Singh. Authors acknowledge the facilities developed under Program Support to Indian Institute of Advanced Research (IIAR) sponsored by the Department of Biotechnology (DBT), Gov't. of India and Russian Fund for Basic Research Russia–India grant number RFBR 11-04-92697-IND-a, RFBR grant number 14-04-01323-a, and Russian Ministry of Education grant number RFMEFI60714X0067 to Dr. Peter Chumakov. Authors also acknowledge the DBT-MSUB-ILSPARE program of the Department of Biochemistry, The MS University of Baroda sponsored by DBT Govt. of India. This work constitutes the Ph.D. thesis of Kritarth Singh. Authors acknowledge the research fellowships from the Council of Scientific and Industrial Research (CSIR), Government of India to Khyati Bhatelia, Dhanendra Tomar, University Grant Commission (UGC), Government of India to Kritarth Singh, Lakshmi Sripada, Indian Council of Medical Research (ICMR), Govt. of India to Arun K Singh, and DST Young Scientist Fellowship to Dr. Rochika Singh.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2015.01.016.

References

[1] D.H. Dapito, A. Mencin, G.Y. Gwak, J.P. Pradere, M.K. Jang, I. Mederacke, J.M. Caviglia, H. Khiabanian, A. Adeyemi, R. Bataller, J.H. Lefkowitch, M. Bower, R. Friedman, R.B. Sartor, R. Rabadan, R.F. Schwabe, Promotion of hepatocellular carcinoma by the intestinal microbiota and TLR4, Cancer Cell 21 (2012) 504–516.
[2] F.M. Robertson, M. Bondy, W. Yang, H. Yamauchi, S. Wiggins, S. Kamrudin, S. Krishnamurthy, H. Le-Petross, L. Bidaut, A.N. Player, S.H. Barsky, W.A. Woodward, T. Buchholz, A. Lucci, N.T. Ueno, M. Cristofanilli, Inflammatory breast cancer: the disease, the biology, the treatment, CA Cancer J. Clin. 60 (2010) 351–375.
[3] J. Terzic, S. Grivennikov, E. Karin, M. Karin, Inflammation and colon cancer, Gastroenterology 138 (2010) 2101–2114 (e2105).
[4] N. Uemura, S. Okamoto, S. Yamamoto, N. Matsumura, S. Yamaguchi, M. Yamakido, K. Taniyama, N. Sasaki, R.J. Schlemper, Helicobacter pylori infection and the development of gastric cancer, N. Engl. J. Med. 345 (2001) 784–789.
[5] B.E. Lippitz, Cytokine patterns in patients with cancer: a systematic review, Lancet Oncol. 14 (2013) e218–e228.
[6] T. Ashizawa, R. Okada, Y. Suzuki, M. Takagi, T. Yamazaki, T. Sumi, T. Aoki, S. Ohnuma, T. Aoki, Clinical significance of interleukin-6 (IL-6) in the spread of gastric cancer: role of IL-6 as a prognostic factor, Gastric Cancer Off. J. Int. Gastric Cancer Assoc. Japan. Gastric Cancer Assoc. 8 (2005) 124–131.
[7] M. Ikeguchi, T. Hatada, M. Yamamoto, T. Miyake, T. Matsunaga, Y. Fukumoto, Y. Yamada, K. Fukuda, H. Saito, S. Tatebe, Serum interleukin-6 and -10 levels in patients with gastric cancer, Gastric Cancer Off. J. Int. Gastric Cancer Assoc. Japan. Gastric Cancer Assoc. 12 (2009) 95–100.
[8] T. Kawabata, T. Ichikura, T. Majima, S. Seki, K. Chochi, E. Takayama, H. Hiraide, H. Mochizuki, Preoperative serum interleukin-18 level as a postoperative prognostic marker in patients with gastric carcinoma, Cancer 92 (2001) 2050–2055.
[9] O. Kayacan, D. Karnak, S. Beder, E. Gullu, H. Tutkak, F.C. Senler, D. Koksal, Impact of TNF-alpha and IL-6 levels on development of cachexia in newly diagnosed NSCLC patients, Am. J. Clin. Oncol. 29 (2006) 328–335.
[10] L. Kozlowski, I. Zakrzewska, P. Tokajuk, M.Z. Wojtukiewicz, Concentration of interleukin-6 (IL-6), interleukin-8 (IL-8) and interleukin-10 (IL-10) in blood serum of breast cancer patients, Rocz. Akad. Med. Bialymst. 48 (2003) 82–84.
[11] J.C. Lee, K.M. Lee, D.W. Kim, D.S. Heo, Elevated TGF-beta1 secretion and down-modulation of NKG2D underlies impaired NK cytotoxicity in cancer patients, J. Immunol. 172 (2004) 7335–7340.
[12] Y. Lin, S. Kikuchi, Y. Obata, K. Yagyu, C. Tokyo, Research group on prevention of gastric, serum levels of transforming growth factor beta1 are significantly correlated with venous invasion in patients with gastric cancer, J. Gastroenterol. Hepatol. 21 (2006) 432–437.
[13] N.I. Nikiteas, N. Tzanakis, M. Gazouli, G. Rallis, K. Daniilidis, G. Theodoropoulos, A. Kostakis, G. Peros, Serum IL-6, TNFalpha and CRP levels in Greek colorectal cancer patients: prognostic implications, World J. Gastroenterol. WJG 11 (2005) 1639–1643.
[14] Y. Ren, R.T. Poon, H.T. Tsui, W.H. Chen, Z. Li, C. Lau, W.C. Yu, S.T. Fan, Interleukin-8 serum levels in patients with hepatocellular carcinoma: correlations with clinicopathological features and prognosis, Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res. 9 (2003) 5996–6001.
[15] K.S. Shim, K.H. Kim, W.S. Han, E.B. Park, Elevated serum levels of transforming growth factor-beta1 in patients with colorectal carcinoma: its association with tumor progression and its significant decrease after curative surgical resection, Cancer 85 (1999) 554–561.
[16] S.I. Grivennikov, F.R. Greten, M. Karin, Immunity, inflammation, and cancer, Cell 140 (2010) 883–899.
[17] W.W. Lin, M. Karin, A cytokine-mediated link between innate immunity, inflammation, and cancer, J. Clin. Invest. 117 (2007) 1175–1183.
[18] O. Micheau, J. Tschopp, Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes, Cell 114 (2003) 181–190.
[19] L. Wang, F. Du, X. Wang, TNF-alpha induces two distinct caspase-8 activation pathways, Cell 133 (2008) 693–703.
[20] A. Benchoua, C. Couriaud, C. Guegan, L. Tartier, P. Couvert, G. Friocourt, J. Chelly, J. Menissier-de Murcia, B. Onteniente, Active caspase-8 translocates into the nucleus of apoptotic cells to inactivate poly(ADP-ribose) polymerase-2, J. Biol. Chem. 277 (2002) 34217–34222.
[21] L. Scorrano, Caspase-8 goes cardiolipin: a new platform to provide mitochondria with microdomains of apoptotic signals? J. Cell Biol. 183 (2008) 579–581.
[22] D. Chandra, G. Choy, X. Deng, B. Bhatia, P. Daniel, D.G. Tang, Association of active Caspase 8 with the mitochondrial membrane during apoptosis: potential roles in cleaving BAP31 and Caspase 3 and mediating mitochondrion–endoplasmic reticulum cross talk in etoposide-induced cell death, Mol. Cell. Biol. 24 (2004) 6592–6607.
[23] J.J. Kim, S.B. Lee, J.K. Park, Y.D. Yoo, TNF-alpha-induced ROS production triggering apoptosis is directly linked to Romo1 and Bcl-X(L), Cell Death Differ. 17 (2010) 1420–1434.
[24] H. Nohl, L. Gille, K. Staniek, Intracellular generation of reactive oxygen species by mitochondria, Biochem. Pharmacol. 69 (2005) 719–723.
[25] E. Gottlieb, M.G. Vander Heiden, C.B. Thompson, Bcl-x(L) prevents the initial decrease in mitochondrial membrane potential and subsequent reactive oxygen species production during tumor necrosis factor alpha-induced apoptosis, Mol. Cell. Biol. 20 (2000) 5680–5689.
[26] D.S. Straus, TNFalpha and IL-17 cooperatively stimulate glucose metabolism and growth factor production in human colorectal cancer cells, Mol. Cancer 12 (2013) 78.
[27] D. Arnoult, F. Soares, I. Tattoli, S.E. Girardin, Mitochondria in innate immunity, EMBO Rep. 12 (2011) 901–910.

[28] R.B. Seth, L. Sun, C.K. Ea, Z.J. Chen, Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3, Cell 122 (2005) 669–682.
[29] Y. Zhao, X. Sun, X. Nie, L. Sun, T.S. Tang, D. Chen, Q. Sun, COX5B regulates MAVS-mediated antiviral signaling through interaction with ATG5 and repressing ROS production, PLoS Pathog. 8 (2012) e1003086.
[30] H. Ishikawa, G.N. Barber, STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling, Nature 455 (2008) 674–678.
[31] K. Bhatelia, A. Singh, D. Tomar, K. Singh, L. Sripada, M. Chagtoo, P. Prajapati, R. Singh, M.M. Godbole, R. Singh, Antiviral signaling protein MITA acts as a tumor suppressor in breast cancer by regulating NF-kappaB induced cell death, Biochim. Biophys. Acta 1842 (2014) 144–153.
[32] C.B. Moore, D.T. Bergstralh, J.A. Duncan, Y. Lei, T.E. Morrison, A.G. Zimmermann, M.A. Accavitti-Loper, V.J. Madden, L. Sun, Z. Ye, J.D. Lich, M.T. Heise, Z. Chen, J.P. Ting, NLRX1 is a regulator of mitochondrial antiviral immunity, Nature 451 (2008) 573–577.
[33] X. Xia, J. Cui, H.Y. Wang, L. Zhu, S. Matsueda, Q. Wang, X. Yang, J. Hong, Z. Songyang, Z.J. Chen, R.F. Wang, NLRX1 negatively regulates TLR-induced NF-kappaB signaling by targeting TRAF6 and IKK, Immunity 34 (2011) 843–853.
[34] L. Sripada, D. Tomar, P. Prajapati, R. Singh, A.K. Singh, R. Singh, Systematic analysis of small RNAs associated with human mitochondria by deep sequencing: detailed analysis of mitochondrial associated miRNA, PLoS ONE 7 (2012) e44873.
[35] D. Tomar, P. Prajapati, L. Sripada, K. Singh, R. Singh, A.K. Singh, R. Singh, TRIM13 regulates Caspase-8 ubiquitination, translocation to autophagosomes and activation during ER stress induced cell death, Biochim. Biophys. Acta 1833 (2013) 3134–3144.
[36] T. Cheng, J. Sudderth, C. Yang, A.R. Mullen, E.S. Jin, J.M. Mates, R.J. DeBerardinis, Pyruvate carboxylase is required for glutamine-independent growth of tumor cells, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 8674–8679.
[37] D. D'Amours, F.R. Sallmann, V.M. Dixit, G.G. Poirier, Gain-of-function of poly(ADP-ribose) polymerase-1 upon cleavage by apoptotic proteases: implications for apoptosis, J. Cell Sci. 114 (2001) 3771–3778.
[38] F. Soares, I. Tattoli, M.A. Rahman, S.J. Robertson, A. Belcheva, D. Liu, C. Streutker, S. Winer, D.A. Winer, A. Martin, D.J. Philpott, D. Arnoult, S.E. Girardin, The mitochondrial protein NLRX1 controls the balance between extrinsic and intrinsic apoptosis, J. Biol. Chem. 289 (2014) 19317–19330.
[39] F. Gonzalvez, D. Lawrence, B. Yang, S. Yee, R. Pitti, S. Marsters, V.C. Pham, J.P. Stephan, J. Lill, A. Ashkenazi, TRAF2 sets a threshold for extrinsic apoptosis by tagging Caspase-8 with a ubiquitin shutoff timer, Mol. Cell 48 (2012) 888–899.
[40] Z. Jin, Y. Li, R. Pitti, D. Lawrence, V.C. Pham, J.R. Lill, A. Ashkenazi, Cullin3-based polyubiquitination and p62-dependent aggregation of Caspase-8 mediate extrinsic apoptosis signaling, Cell 137 (2009) 721–735.
[41] J. Huai, F.N. Vogtle, L. Jockel, Y. Li, T. Kiefer, J.E. Ricci, C. Borner, TNFalpha-induced lysosomal membrane permeability is downstream of MOMP and triggered by caspase-mediated NDUFS1 cleavage and ROS formation, J. Cell Sci. 126 (2013) 4015–4025.
[42] R.B. Hamanaka, N.S. Chandel, Mitochondrial reactive oxygen species regulate cellular signaling and dictate biological outcomes, Trends Biochem. Sci. 35 (2010) 505–513.
[43] F.J. Lopez-Hernandez, M.A. Ortiz, Y. Bayon, F.J. Piedrafita, Z-FA-fmk inhibits effector caspases but not initiator caspases 8 and 10, and demonstrates that novel anticancer retinoid-related molecules induce apoptosis via the intrinsic pathway, Mol. Cancer Ther. 2 (2003) 255–263.
[44] T. Geiger, A. Wehner, C. Schaab, J. Cox, M. Mann, Comparative proteomic analysis of eleven common cell lines reveals ubiquitous but varying expression of most proteins, Mol. Cell. Proteomics MCP 11 (2012) 014050 (M111).
[45] D. Hanahan, R.A. Weinberg, Hallmarks of cancer: the next generation, Cell 144 (2011) 646–674.
[46] I. Tattoli, L.A. Carneiro, M. Jehanno, J.G. Magalhaes, Y. Shu, D.J. Philpott, D. Arnoult, S.E. Girardin, NLRX1 is a mitochondrial NOD-like receptor that amplifies NF-kappaB and JNK pathways by inducing reactive oxygen species production, EMBO Rep. 9 (2008) 293–300.
[47] Z.T. Schug, F. Gonzalvez, R.H. Houtkooper, F.M. Vaz, E. Gottlieb, BID is cleaved by caspase-8 within a native complex on the mitochondrial membrane, Cell Death Differ. 18 (2011) 538–548.
[48] N.J. Lanning, B.D. Looyenga, A.L. Kauffman, N.M. Niemi, J. Sudderth, R.J. DeBerardinis, J.P. MacKeigan, A mitochondrial RNAi screen defines cellular bioenergetic determinants and identifies an adenylate kinase as a key regulator of ATP levels, Cell Rep. 7 (2014) 907–917.
[49] D. Arnoult, F. Soares, I. Tattoli, C. Castanier, D.J. Philpott, S.E. Girardin, An N-terminal addressing sequence targets NLRX1 to the mitochondrial matrix, J. Cell Sci. 122 (2009) 3161–3168.
[50] G. He, M. Karin, NF-kappaB and STAT3 — key players in liver inflammation and cancer, Cell Res. 21 (2011) 159–168.
[51] P.P. Hsu, D.M. Sabatini, Cancer cell metabolism: Warburg and beyond, Cell 134 (2008) 703–707.
[52] D.W. Zhang, J. Shao, J. Lin, N. Zhang, B.J. Lu, S.C. Lin, M.Q. Dong, J. Han, RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis, Science 325 (2009) 332–336.
[53] P. Maycotte, C.M. Gearheart, R. Barnard, S. Aryal, J.M. Mulcahy Levy, S.P. Fosmire, R.J. Hansen, M.J. Morgan, C.C. Porter, D.L. Gustafson, A. Thorburn, STAT3-mediated autophagy dependence identifies subtypes of breast cancer where autophagy inhibition can be efficacious, Cancer Res. 74 (2014) 2579–2590.
[54] Y. Lei, H. Wen, Y. Yu, D.J. Taxman, L. Zhang, D.G. Widman, K.V. Swanson, K.W. Wen, B. Damania, C.B. Moore, P.M. Giguere, D.P. Siderovski, J. Hiscott, B. Razani, C.F. Semenkovich, X. Chen, J.P. Ting, The mitochondrial proteins NLRX1 and TUFM form a complex that regulates type I interferon and autophagy, Immunity 36 (2012) 933–946.
[55] A.S. Cleary, T.L. Leonard, S.A. Gestl, E.J. Gunther, Tumour cell heterogeneity maintained by cooperating subclones in Wnt-driven mammary cancers, Nature 508 (2014) 113–117.
[56] K. Bhatelia, K. Singh, R. Singh, TLRs: Linking inflammation and breast cancer, Cell. Signal. 26 (2014) 2350–2357.